Presence and Distribution of Cholinergic Nerves in Rat Mediastinal Brown Adipose Tissue

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SUMMARY  Brown adipose tissue (BAT) is richly provided with sympathetic noradrenergic nerves but is believed to lack a parasympathetic nerve supply. Acetylcholine is the predominant transmitter of postganglionic parasympathetic nerves. The vesicular acetylcholine transporter (VACt) resides in synaptic vesicles of cholinergic nerve terminals and is used as a marker for peripheral cholinergic nerves. We sought cholinergic nerves in rat BAT using VACt immunohistochemistry (IHC) on cryosections of interscapular, cervical, mediastinal, and perirenal depots. Mediastinal BAT was the sole depot provided with putative parasympathetic perivascular and parenchymal cholinergic nerves. The absence of vasoactive intestinal peptide-positive nerves suggested their nature as pure cholinergic fibers. By confocal microscopy, both cholinergic and noradrenergic nerves were detected in mediastinal BAT. Cold exposure and fasting led to increased density of VACt-positive fibers and of noradrenergic sympathetic nerves at morphometry. The unexpected double innervation of mediastinal BAT may explain the inhibitory influence on thermogenesis observed after systemic injection of muscarinic antagonists in rats, and raises questions about the physiological role of its cholinergic nerve supply. (J Histochem Cytochem 52:923–930, 2004)

KEY WORDS  cholinergic nerves  parasympathetic system  brown adipose tissue  noradrenergic nerves  fasting  cold exposure  thermoneutrality  rat
Peripheral postganglionic cholinergic nerves are held to belong to the parasympathetic nervous system. The aim of the present work was therefore to establish whether rat subcutaneous and visceral BAT depots are provided with cholinergic, putatively parasympathetic, nerves. To address this question, we investigated by IHC the occurrence and distribution of VACHT-positive nerves in interscapular, cervical, mediastinal, and perirenal BAT depots of rats kept at different environmental temperatures and of fasted rats. The results showed that only mediastinal BAT is provided with cholinergic nerves and that they are modulated by both cold and fasting.

Materials and Methods

Animals and Tissues

Male Sprague–Dawley rats 9 weeks of age were obtained from Morini Laboratories (S. Polo d’Enza, Italy). They were singly caged and randomly assigned to the following four experimental groups: five rats with free access to food (65% carbohydrates, 11% fat, 24% protein, w/w) and water were kept at 22°C (control group); five animals were kept at 4°C (cold-exposed group); five rats were studied for each experimental condition, and an amount of mediastinal BAT containing 500 brown adipocytes was measured for each experimental condition. The number of nerve spots and the area (\(\mu\text{m}^2\)) occupied by brown adipocytes was measured for each experimental condition. The number of nerve spots and the area (\(\mu\text{m}^2\)) occupied by brown adipocytes was measured for each experimental condition. The number of nerve spots and the area (\(\mu\text{m}^2\)) occupied by brown adipocytes was measured for each experimental condition. The number of nerve spots and the area (\(\mu\text{m}^2\)) occupied by brown adipocytes was measured for each experimental condition.
(polyclonal rabbit; Sigma) at a concentration of 1.3 μg/ml and the primary antibody against TH (polyclonal sheep; Chemicon) at a concentration of 0.3 μg/ml, washed twice with PB (15 min each), and incubated in 1:100 v/v FITC donkey anti-rabbit (Jackson) and TRITC donkey anti-sheep (Jackson) antibody in PB for 30 min at RT. Sections were subsequently washed twice with PB, air-dried, and coverslipped using the Vectashield mounting medium (Vector). Fluorescence was detected with a BioRad (Hercules, CA) Microradiance confocal laser scanning microscope equipped with an argon and He/Ne mixed gas laser. FITC and TRITC were excited with the 488- and 543-nm lines, respectively, imaged separately (emissions were separated using 515/30- and 570-nm filters), and then merged using the LaserSharp Processing Bio-Rad software (version 3.2). Sections were viewed in an Eclipse E600 Nikon microscope with a ×60 plan apochromat objective and 1.4 numerical aperture. The images (512 × 512 pixels) were then obtained sequentially from two channels using a confocal pinhole of 2–3. The images were stored as TIFF files. Brightness and contrast of the final images were adjusted using the Photoshop 6 software (Adobe Systems).

Results

Mediastinal BAT Is the Only Brown Fat Depot Provided with Cholinergic Nerves

In rats, mediastinal BAT is found in the upper and posterior mediastinum. It is composed of many different-sized lobules lying among the large mediastinal blood vessels, heart, trachea, esophagus, and descending aorta. In control rats, VAChT-positive nerves provided surrounding viscera. Occasionally they were also found in the mediastinal BAT lobules, sometimes around blood vessels, more often arteries (Figure 1A), but more frequently running among brown adipocytes in the parenchyma (Figure 1B). The density of VAChT-positive nerves varied among BAT lobules regardless of their size or anatomic position. The origin of these cholinergic nerves was probably the postganglionic neurons contained in the parasympathetic ganglia located close to the mediastinal viscera (Figure 2A). Cholinergic nerves usually co-localize VIP (Lindh and Hökfelt 1990). Nevertheless, with a specific antibody against VIP (Figure 2B), VIP-positive nerves were never found in mediastinal BAT in control rats or in any other experimental condition investigated (see below). Double labeling experiments in mediastinal BAT sections showed the presence of both cholinergic and noradrenergic nerves, which ran parallel and in close proximity to one another both around blood vessels and in the parenchyma under all conditions examined (Figure 3).

VAChT-positive nerves were never found in interscapular, cervical, and perirenal BAT depots in control animals or in any of the experimental conditions investigated (see below). Mediastinal BAT is therefore the only BAT depot in rats to be provided with both sympathetic noradrenergic and putatively parasympathetic cholinergic innervation.

Cholinergic Nerve Density Increases After Cold Exposure in Mediastinal BAT

To verify whether cold, the physiological stimulus for BAT heat production and recruitment (Trayhurn and Nicholls 1986), modulates the cholinergic nerve supply to mediastinal fat, we compared by IHC and morphological analysis the density of TH- and VAChT-positive nerves in mediastinal BAT in rats kept at temperatures close to thermoneutrality (28°C) and in animals exposed to low temperatures (4°C) for 2 days. As expected, TH-positive nerves were sparse in the former animals, whereas their density significantly increased after cold exposure (Figures 4A and 4B). Notably, very few vascular and parenchymal VAChT-positive nerves were found in mediastinal BAT in rats kept in a thermoneutral condition. After cold exposure, VAChT immunoreactivity was more evident at perivascular sites and the density of VAChT-positive nerves among brown adipocytes increased significantly (Figures 4A and 4B).

Cholinergic Nerve Density Increases During Fasting in the Mediastinal BAT

Fasting is associated with complex metabolic and morphological changes in BAT (Cinti 1999). Food reduction and starvation are generally believed to inhibit BAT heat production (Trayhurn and Jennings 1988), probably to save lipids for the general metabolism, but brown adipocytes may retain the ability to produce heat to maintain a constant body temperature under certain conditions (Champigny and Ricquier 1990). Compared with controls, rats fasted for 4 or 5 days (which showed a homogeneous weight reduction of ~25 ± 1.5%) exhibited a more evident TH immunoreactivity around blood vessels and a significant increase in parenchymal noradrenergic nerve density (Figures 4C and 4D). In parallel, vascular and parenchymal VAChT-positive nerves increased, albeit not significantly, largely because of the extreme variability of their density among the lobules (Figures 4C and 4D).

Discussion

Among the four larger BAT depots of rats (interscapular, cervical, mediastinal, and perirenal), only mediastinal brown fat lobules are provided with cholinergic nerves. In this study we used VAChT to detect cholinergic nerves (Arvidsson et al. 1997). VAChT belongs to the family of vesicular transporters, whose function is to concentrate neurotransmitters into synaptic vesicles through proton exchange (Bravo and Parsons...
Figure 1  Vesicular acetylcholine transporter IHC in rat mediastinal brown adipose tissue. In control animals, cholinergic nerves are found in the adventitia of intralobular blood vessels (A) and in the parenchyma, among the brown adipocytes (B). The spots indicating specific immunostaining (arrows in B) depict the nerve pathway and probably represent the releasing site of the neurotransmitter along the axon. A, artery. Bar = 25 μm.
Figure 2  (A) Vesicular acetylcholine transporter (VACHT) IHC in rat mediastinum. A parasympathetic ganglion is located near an artery (A) and a vein (V). It contains cholinergic neurons showing weak cytoplasmic VACHT immunoreactivity. The spots of immunoreactivity around the cell bodies are terminals of preganglionic cholinergic nerves. (B) Vasoactive intestinal peptide IHC in rat intestine. Many positive nerves are found in the mucosa. (Insets) Enlargements of the corresponding framed areas. Bars: A,B = 80 μm; insets = 25 μm.
Figure 3  Indirect immunofluorescence in rat mediastinal brown adipose tissue. Confocal microscopy reveals the presence in the same section of both noradrenergic (TH, red) and cholinergic (VACHT, green) nerves at perivascular (left panels) and parenchymal (right panels) sites. Lu, lumen of an artery. Bar = 15 μm.
In cholinergic neurons, acetylcholine is synthesized in the cytoplasm of nerve endings by choline acetyl transferase and is transported by VACHT into synaptic vesicles, where it is stored until release (Prado et al. 2002). Therefore, by detecting the presynaptic pool of cholinergic vesicles, VACHT IHC allows visualization of the sites of neurotransmitter release along axons (Schafer et al. 1995; Gilmor et al. 1996; Arvidsson et al. 1997; Schafer et al. 1998). In mediastinal BAT, VACHT-positive nerves were observed at peri-vascular sites and, more often, among brown adipocytes. This pattern of immunostaining lends support to the hypothesis that acetylcholine is indeed released by both perivascular and parenchymal cholinergic nerves and that it exerts an effect on blood vessels and brown adipocytes.

A possible cholinergic control over mammalian thermogenesis has been hypothesized. In rats, injection of the muscarinic receptor antagonist atropine sulfate enhances the acute thermogenic response to a meal, an effect that is particularly evident in genetically obese Zucker rats, which normally show defective diet-induced thermogenesis (Rothwell et al. 1981). Furthermore, injection of atropine or surgical vagotomy also prevents metabolic rate decline after glucopenia induced by central or peripheral injections of 2-deoxy-D-glucose (Shiraishi and Mager 1980; Rothwell et al. 1981). These findings suggest that, by acting through muscarinic receptors, acetylcholine exerts an inhibitory influence on thermogenesis, particularly in obese rodents. Considering these data, Bryant et al. (1983) attempted to determine whether rat BAT was provided with cholinergic nerves by measuring tissue acetylcholine levels and acetylcholinesterase activity. Since the study was conducted on interscapular BAT which, in line with our data, was completely devoid of cholinergic innervation, the authors concluded that there was no evidence of parasympathetic innervation in rat BAT. On the contrary, our data suggest that a putative inhibitory influence on thermogenesis exerted by acetylcholine may take place in mediastinal BAT, where acetylcholine released by parenchymal cholinergic axons may act directly on brown adipocytes or, possibly, may exert prejunctional inhibition of norepinephrine release from adjacent sympathetic nerves. Of course, additional central and/or hormonal effects on BAT thermogenesis after experimental manipulation of the cholinergic system cannot be excluded.

Cold exposure and fasting led to increased density of VACHT-positive fibers in addition to noradrenergic sympathetic nerves. Therefore, any influence of cholinergic nerves on brown adipocyte thermogenesis and metabolism increases during cold-dependent and fast-dependent heat production and/or lipid secretion.

Acetylcholine is usually found in the parasympathetic system throughout the body. Therefore, the cholinergic nerves found in mediastinal BAT are probably postganglionic parasympathetic nerves pertaining to the vagal nerve and arising from the many para-
sympathetic ganglia located close to the mediastinal viscera. Nevertheless, sympathetic postganglionic cholinergic nerves have been described in sweat glands (Landis and Keefe 1983; Leblanc and Landis 1986), arterial microvasculature of skeletal muscle (Bolme and Fuxe 1970), and periosteum (Asmus et al. 2000). Therefore, it cannot be excluded that the cholinergic axons supplying mediastial brown fat lobules could derive from the cholinergic neurons of the sympathetic thoracic chain (Schafer et al. 1998).

We did not find cholinergic innervation in interscapular, cervical, and perirenal BAT depots. However, it should be noted that non-cholinergic nitric oxide-, VIP-, and/or calcitonin gene-related peptide-containing parasympathetic nerves have recently been described in human airways (van der Velden and Hulsman 1999) and in rat heart (Onuoha et al. 1999). Therefore, our results do not exclude a non-cholinergic parasympathetic nerve supply to other BAT depots. Of note is that a dual sympathetic and parasympathetic innervation has recently been hypothesized for rat white adipose tissue (Kreier et al. 2002).

On the other hand, if further studies were to exclude the presence of a parasympathetic nerve supply to the other BAT depots, mediastial BAT would be the sole BAT depot provided with both a sympathetic and a parasympathetic nerve supply. Such double control may be connected with a closer and more subtle modulation of brown adipocyte metabolism and heat production in the mediastinum, possibly in a still unknown relationship to cardiac activity.

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Literature Cited


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